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Isolation of Purified Oocyst Walls and Sporocysts from *Toxoplasma gondii*

WILLIAM V. EVERSON,[a] MICHAEL W. WARE,[a] J. P. DUBEY[b] and H. D. ALAN LINDQUIST[a]

[a]*Microbiological and Chemical Exposure Assessment Research Division, National Exposure Research Laboratory, United States Environmental Protection Agency, Cincinnati, Ohio 45268, USA, and*

[b]*Parasite Biology and Epidemiology Laboratory, United States Department of Agrigulture, Agricultural Research Service, Animal and Natural Resources Institute, Beltsville, Maryland 20705-2350, USA*

ABSTRACT. *Toxoplasma gondii* oocysts are environmentally resistant and can infect virtually all warm-blooded hosts, including humans and livestock. Little is known about the biochemical basis for this resistance of oocysts, and mechanism for excystation of *T. gondii* sporozoites. The objective of the present study was to evaluate different methods (mechanical fragmentation, gradients, flow cytometry) to separate and purify *T. gondii* oocyst walls and sporocysts. Oocyst walls were successfully separated and purified using iodixanol gradients. Sporocysts were successfully separated and purified using iodixanol and Percoll gradients. Purification was also achieved by flow cytometry. Flow cytometry with fluorescence-activated cell sorting (FACS) yielded analytical quantities of oocyst walls and intact sporocysts. Flow cytometry with FACS also proved useful for quantitation of purity obtained following iodixanol gradient fractionation. Methods reported in this paper will be useful for analytical purposes, such as proteomic analysis of components unique to this life cycle stage, development of detection methods, or excystation studies.

Key Words. Flow cytometry, fluorescence-activated cell sorting (FACS), gradient sedimentation, iodixanol.

Toxoplasmosis is a serious disease in pregnancy and also an opportunistic disease of great concern to patients with impaired immune systems (Cohen 1999; Dubey 2000). Humans become infected via any of the three infectious stages of *T. gondii*: from tachyzoites transplacentally (Cohen 1999), from tissue cysts by eating uncooked meat (Dubey et al. 1995), and from oocysts by ingesting food and water contaminated with infected cat feces (Aramini et al. 1999; Bowie et al. 1997; Coutinho, Lobo, and Dutra 1982; Dubey 1998; Eng et al. 1999; Frenkel, Ruiz, and Chinchilla 1975; Ito et al. 1975; Ruiz, Frenkel, and Cerdas 1973; Were et al. 1999). Little is known of the biology of *T. gondii* except that it is infectious to virtually any warm-blooded host and that these parasites are highly resistant to environmental influences. Nothing is known of the biochemical basis for this environmental resistance. These studies have not been performed in part because it is hazardous to produce and purify *T. gondii* oocysts.

Toxoplasma gondii consists of an oocyst wall surrounding two sporocysts, each of which is comprised of a sporocyst wall surrounding four sporozoites (Dubey, Lindsay, and Speer 1998; Speer, Clark, and Dubey 1998). In this paper, we describe a method for isolation of oocyst walls that can serve as enriched starting material for development of a detection method and analysis of the components. We (data not shown) and others have had difficulty in developing antibodies against whole oocysts (Kasper and Ware 1985). In addition, this method provides materials that can be used for analysis of the oocyst wall components, excystation studies, and identification of components that are specific to this life cycle stage, by use of proteomic analysis or other similar methods.

MATERIALS AND METHODS

Two sequential steps were used for isolation of oocyst components. Oocysts were first fragmented mechanically and oocyst walls and sporocysts were then separated by different methods. Several methods were compared for separation and isolation of oocyst fragments: use of Percoll-based sedimentation (gravity or centrifugation); use of iodixanol gradient sedimentation; and flow cytometry.

Source of oocysts. *Toxoplasma gondii* oocysts of the VEG strain were obtained in feces of cats fed tissues cysts and purified as described (Dubey et al. 1995; Speer, Clark, and Dubey 1998). Oocysts were sporulated in 2% H_2SO_4 on a shaker, and then stored at 4 °C for 1–12 mo until used. Immediately prior to use, aliquots containing between 5×10^6 to 2×10^8 oocysts suspended in 2% H_2SO_4 were neutralized by addition of 3/5 of the original vol. of a 1 N NaOH solution (v/v), and washed by addition of 50 ml of PBS for each 1–2 ml of starting material. Oocysts were recovered as a pellet by centrifugation at 2,000 *g* for 10 min, and resuspended in a small vol. of PBS to a concentration of 1×10^6 to 2×10^7 oocysts/ ml.

Quantitation of the purity of fractions. Since the goal of these studies was to obtain purified walls and other fractions, purity of fractions was measured rather than quantitative recovery. This was done by manual counting of aliquots, which had been spotted onto microscope slides and cover-slipped. To quantitate fragmentation, a small aliquot of the pooled supernatants was spotted onto slides and subjected to manual counting and classification. All objects larger than 1 μm were counted and classified for a total of 100–500 objects. Differential interference contrast microscopy (DIC), phase microscopy, or fluorescent detection following Nile Red staining (9-diethylamino-5H-benzo{alpha}phrenoxazine-5-one, Molecular Probes, Eugene, Oregon) was used to determine and discriminate wall fragments from intact or broken oocysts, to detect sporocysts still attached to wall fragments, and to determine the number of sporocysts broken during fragmentation. Objects were classified as follows: 1) intact oocysts (i.e. oocyst containing 2 sporocysts with no evident break in the oocyst wall); 2) intact oocysts with no sporocysts (e.g. non-sporulated); 3) partially fragmented oocysts (i.e. evidence of a wall broken by discontinuity in the Nile Red fluorescence, but one or both sporocysts still visible within or adherent to the oocyst wall); 4) intact oocyst walls (a large wall fragment remaining intact as evidenced by the apparent equivalent relative diameter in one dimension as the intact oocyst); 5) intact sporocysts (no visible break in the continuity of the sporocyst wall Nile Red fluorescence); 6) broken sporocysts (evidenced by a break, i.e. a discontinuity in the Nile Red fluorescence of the sporocyst wall); 7) small debris (debris larger than 1 μm).

In some instances, the purity of the fractions collected following separation by gradient methods was verified by fluorescence-activated cell sorting (FACS). The wall fragments and sporocysts present within a given gradient fraction sample were automatically tabulated during sorting and expressed as percentages of the total count of oocyst walls and sporocysts in the two sort regions.

Nile Red staining and fluorescence. Nile Red exhibits fluorescence in a hydrophobic environment. This fluorescence is quenched in aqueous environments (Greenspan and Fowler 1985). Nile Red has not been previously used as a vital stain

Corresponding Author: A. Lindquist–Telephone number: 513-569-7192; FAX number: 513-569-7117; E-mail: lindquist.alan@epa.gov

for oocysts, but has recently been used to stain lipid droplets within tachyzoites in infected cells (Sonda et al. 2001). The oocyst walls, sporocyst walls, and intracellular contents provided an environment that induced fluorescence, while the aqueous media remained relatively non-fluorescent, allowing fluorescence microscopy and FACS without a further wash step. For microscopic analysis, neither glycerol nor other hydrophobic chemicals were used because they caused a background fluorescence and leached Nile Red from the walls and membranes.

Solutions containing oocysts or fragments, and gradient fractions were incubated with Nile Red in PBS at concentrations ranging from 0.1–1 μg /ml for 5 min.

Mechanical fragmentation of oocysts. Use of glass beads with vortexing to mechanically fragment oocysts has been previously reported by other laboratories (Speer et al. 1995; Speer et al. 1997). It was determined by Speer et al. (1997) that optimal fragmentation was dependent on tube geometry. Since we selected 1.5-ml microfuge tubes, the first step in the procedure was the optimization of fragmentation conditions. We varied bead vol., total vol., oocyst concentrations, and fragmentation time.

We evaluated total volume ranging from 300–1500 μl, glass bead volume (Sigma G8772, 425–600 μ, acid-washed) from 200–1000 μl, oocyst concentrations from 1×10^6 to 1×10^8 per ml and cumulative vortex time from 1–10 min. In some experiments, a small aliquot (~ 5 μl) was removed and examined microscopically to evaluate the fragmentation process after each 30 or 60 sec time increment. After fragmentation, supernatant containing walls and oocysts was transferred to a new tube by careful aspiration. Beads were washed twice by addition of PBS (400 μl), mixed by inversion, and the supernatant was removed. The initial supernatant and the two washes were pooled. These pooled supernatants were the starting material for the three methods of separation of oocyst walls and sporocysts.

The criteria for successful fragmentation were determined through an iterative process combining fragmentation with a separation method. After the separation step (such as centrifugation) was completed, gradient tubes were fractionated by aspiration from the top, and the material in each step gradient, and at each interface, where a visible band was present, was collected by removing layers sequentially and collecting them in separate tubes. Material in each tube was diluted four-fold with PBS and collected by centrifugation. From each pellet, a small aliquot was subjected to microscopic analysis and classification of objects to identify contaminants (e.g. partially fragmented oocysts with the fraction enriched in sporocysts). This was carried out in an iterative process aimed at optimizing separation of sporocysts from oocyst walls, obtaining high purity of fractions, and minimizing contamination by either partially fragmented or excessively fragmented oocyst components.

The following criteria were established from this iterative process: a successful fragmentation contained less than 2% of intact oocysts. More than 95% of sporocysts were free (i.e. no longer associated with oocyst walls) and more than 98% of sporocysts remained intact. To ensure we were not missing fragmentation of sporocysts by the presence of smaller debris, successful fragmentation contained less than 10% small debris as a percentage of total objects counted. Non-sporulated oocysts did not appear to be fragmented under the conditions employed, so they were not counted as part of the intact oocyst fraction, as they neither changed, nor contributed to the oocyst wall or sporocyst pools within fragmented oocysts. They were monitored and counted during separation procedures.

Flow cytometry with FACS sorting, confocal microscopy, and fluorescence microscopy. Flow cytometry and FACS sorting were carried out using a Becton Dickson FACSVantage SE. Use of linear or log gains in the detector channels varied dependent on the detector and parameter being examined, and are shown by the use of log or linear scales in the units shown on axis labels of flow cytometry profiles.

Table 1. Filter sets used in epifluorescence microscopy and comparable wavelengths and filter sets used in fluorescence-activated cell sorting (FACS) or laser scanning confocal microcopy (Confocal) of isolated oocysts and sporocysts of *Toxoplasma gondii.*

Filter set	Instrument	Excitation filter wavelength (nm)	Dichroic beamsplitter (nm)	Emission filter wavelength (nm)
Yellow/green	Microscopy	430–510	>500	>515
	FACS	488	<560	500–560
	Confocal	488	—	490–554
Red	Microscopy	534–558	>565	>590
	FACs	488	>610	608–652

Confocal microscopy was carried out using a MRC 1,000 (BioRad, Hercules, CA) attached to a Zeiss Axiophot microscope equipped with a Zeiss 40×, na 1.3 lens. Two photomultiplier tubes were available for fluorescence; direct transmission was also captured for DIC imaging in the same confocal plane using the TX-1 attachment (BioRad). Images were captured using Comos software (BioRad, v. 7.0a), and processed using Confocal Assistant (BioRad, v. 4.02), Photoshop (Adobe Systems, San Jose, CA, v. 5.0), and ImageJ (NIH, Bethesda, MD, 1.18×). The filter sets for microscopy will be referred to as yellow/green, or red fluorescence, according to the filter sets used for each method (Table 1). Direct transmission images were generated from either the 488- or 568-nm excitation lines of the laser. During collection of images, fluorescence scanning and direct transmission were alternated so that fields in the same focal plane could be compared. This allowed structures that were identifiable by morphology in DIC microscopy to be compared to localized Nile Red fluorescence in the same plane of focus.

Separation of sporocysts using Percoll. Fragmented oocysts were subjected to a variety of gravity and centrifugal methods employing both step and continuous Percoll gradients. Although we were unable to determine conditions for gradient fractions that allowed for clean separation of oocyst walls from other fragments, we were able to isolate fractions enriched in sporocysts. The simplest method for isolation of sporocysts was sedimentation over a Percoll step gradient for 30 min. Percoll step gradients in PBS were prepared by layering 2 ml of the following solutions: 5%, 10%, 15%, 20%, 25%, 30% and 50% (v/v) Percoll into a 15-ml tube, in sucrose buffer (250 mM sucrose containing 15 mM Tris/Cl, pH 7.5). One ml of oocyst fragments in PBS was layered onto the top of the gradient, and tubes were centrifuged at 1,000 *g* for 30 min in a swinging bucket rotor, using a PR 7,000 centrifuge (IEC, Needham Heights, MA) set for slow acceleration and no brake.

Separation of oocyst walls using iodixanol. Fragmented oocysts were diluted in NaCl/hepes buffer (0.85% NaCl, 10 mM hepes, pH 7.4), and combined with a solution of iodixanol (Optiprep™, 5,5′-[(2-hydroxy-1-3-propanediyl)-bis (acetylamino)] [N,N′ bis (2,3-dihydroxypropyl)-2,4,6-triiodo-1,3-benzenecarboxamide], Accurate Chemical & Scientific Corporation, Westbury, NY) to a final concentration ranging from 10–30% (v/v) iodixanol. Solutions were loaded into 11.5-ml polyallomer tubes, capped, and centrifuged in a fixed angle, vTi 80 rotor at 397,000 *g* for 3–16 h using a Beckman-Coulter Optima XL-

100K ultracentrifuge. After centrifugation, fractions of 0.5–1.0 ml were collected by gravity flow from the bottom of the tube after insertion of a capillary tube connected to small tubing, pre-filled with PBS. Approximate densities at which specific fragments banded were calculated by measuring the refractive index, and determining the density from the formula: density (g/ml) = 3.361 × (refractive index) + 3.417 (formula provided by vendor). After collection and pooling, fractions were diluted 5× (v/v) with NaCl/hepes buffer and centrifuged at 2,500 *g* for 10 min to pellet oocyst fragments in the fraction. Pellets were resuspended in a minimum vol. of NaCl/hepes buffer (~ 50 μl) and an aliquot was spotted onto slides and a cover slip was applied. From each fraction, an aliquot was examined using light or fluorescence microscopy to identify gradient fractions that contained walls and to determine purity. Iodixanol gradients in sucrose buffer resulted in cleaner separation of fragments compared to Iodixanol gradients in NaCl/ hepes buffer in the pilot experiments, so iodixanol in sucrose buffer was employed in subsequent procedures. Sporocysts and wall fragments were resolved in different regions of a continuous gradient formed during centrifugation, and were clearly separated using a starting concentration of 15% (v/v) iodixanol in sucrose buffer, following centrifugation at 397,000 *g* for 16 h.

Based on the density range developed in this gradient, we prepared preformed step gradients to cover the range from 2.5% to 30% (v/v) in intervals of 2.5% or 5% (v/v) steps (in several different gradient preparations). We also compared top loading (sedimentation gradients) with bottom loading (e.g. tubes in which the fragmented oocysts were first mixed with iodixanol to a final concentration of 30% (v/v) in sucrose buffer, followed by overlaying with step gradients referred to as flotation gradients). Identical step gradients containing equivalent amounts of material either top-loaded or bottom-loaded were centrifuged in pairs at 1,000 *g*, for 20–100 min. To simplify the step gradients, some of the intermediate gradient steps in which no visible banding was present at the interface between adjacent steps were eliminated.

Flow cytometry and separation by fluorescent-activated cell sorting (FACS). To generate a fluorescence signal, fragmented oocysts were first incubated with Nile Red at a concentration of 1–5 μg/ml for 2 min. Flow cytometry profiles were developed based on differences in forward or side scatter and fluorescence of the oocyst wall vs. sporocysts using the channels as defined in Table 1. Profiles generated from homogeneous populations (oocysts, oocyst walls, or sporocysts) were run separately to aid in generation of sort regions. Regions for sorting were determined empirically by an iterative process of selection of a region that appeared to capture a population, sorting, collection of 1–10,000 objects and analysis by microscopic evaluation.

RESULTS

Determination of optimal conditions for fragmentation of oocysts. To optimize fragmentation, an iterative process was followed by varying fragmentation conditions, microscopic quantitation of the fragments, and separation on step gradients, collection of fractions, and microscopic quantitation of the material in each fraction from each gradient. During this iterative process, we observed that oocyst walls were more easily fragmented than sporocysts. As a check to ensure our fragmentation procedure was not leading to loss of oocyst walls because of loss of ability to distinguish small fragments from other debris, we summed the total number of large wall fragments (i.e. intact oocysts, partially fragmented oocysts with a sporocyst still attached to a wall fragment, and large oocyst wall fragments) and compared this to the sum of sporocysts. If the sum of oocyst walls was more than 2% different from 1/2 the number of sporocysts, we considered that the fragmentation was excessive.

We also found that breaking the oocyst wall open without mechanical separation of sporocyts from the wall was not sufficient to allow walls and sporocysts to separate readily. When a large proportion of sporocysts remained associated with oocyst walls, these tended to sediment in the same location as the wall fragments. But isolated sporocysts were significantly more buoyant than walls, or walls still associated with sporocyts. If fragmentation resulted in sporocyst wall breakage, the sporocyst walls contaminated the oocyst wall fraction.

A summary of our iterative cycles gave the following conditions, which enabled us to routinely meet the established criteria for fragmentation resulting in separation on gradients of oocysts walls from sporocysts: 400 ± 50 μl of glass beads (approximately 600 μg) were combined with 400–700 μl of oocysts in a suspension at a concentration of 1–10 × 10^7 oocysts/ml in each 1.5 ml microfuge tube. Tubes were vortexed at maximum speed for between 2.5–4 min. After 4 min, the percentage of broken sporocysts and small fragments exceeded the established criteria.

Intact oocysts (Fig. 1, 2) fragmented into predominately large wall fragments and sporocysts. The wall fragments (w, Fig. 3, 4) appeared to remain as large fragments and the sporocysts (s, Fig. 3, 4) appeared to be intact. Fluorescence of sporocyst walls and structures within sporozoites simplified identification of material remaining associated with walls for quantitative analysis of free vs. sporocyst-associated walls.

Even under conditions which were optimized to obtain complete fragmentation of oocysts, a few oocysts remained unfragmented. The majority of these unfragmented oocysts were not sporulated, as determined by microscopic examination. There was no discernible sporocyst wall under either DIC microscopy or Nile Red fluorescence microscopy (n-s, Fig. 1, 2).

Isolation of a fraction containing intact sporocysts by separation using Percoll gradients. Percoll gradients did not lead to separation of oocyst walls under the conditions employed. Fractions containing oocyst walls also contained sporocysts, non-sporulated oocysts, and other fragments when a range of gradient concentrations or types was employed to separate fragments, by either gravity sedimentation or centrifugal separation. However, we were able to separate out a fraction enriched in sporocysts. Some sporocysts distributed throughout Percoll gradients, either under the influence of gravity or during centrifugation. A band developed at the buffer to 5% (v/v) Percoll interface, which consisted primarily of sporocysts.

Separation by equilibrium sedimentation using iodixanol. The optimal separation of walls and sporocysts was obtained with step gradients prepared with iodixanol, prepared in sucrose buffer. We were able to separate and collect bands containing highly purified sporocysts or bands containing highly purified oocyst wall fragments.

The banding patterns/positions were compared by visual inspection side-by-side with top- and bottom-loaded tubes after each 20-min interval, to determine the time required for fragments to reach equivalent (steady-state) positions. After a cumulative centrifugation time of 60 min, the fractions sedimented to a stable steady state as indicated by the same qualitative band pattern present in both flotation and sedimentation gradients. No change was evident in the banding pattern after either 1 or 2 additional 20-min increments (i.e. a cumulative centrifugation time of 80- and 100 min). The optimal time of centrifugation to achieve a steady-state pattern of separation of fragments was therefore determined to be one hour. The final conditions for step gradients used for the separation consisted of layers of 2.5-ml fractions of the following solutions: 5, 15, 20,

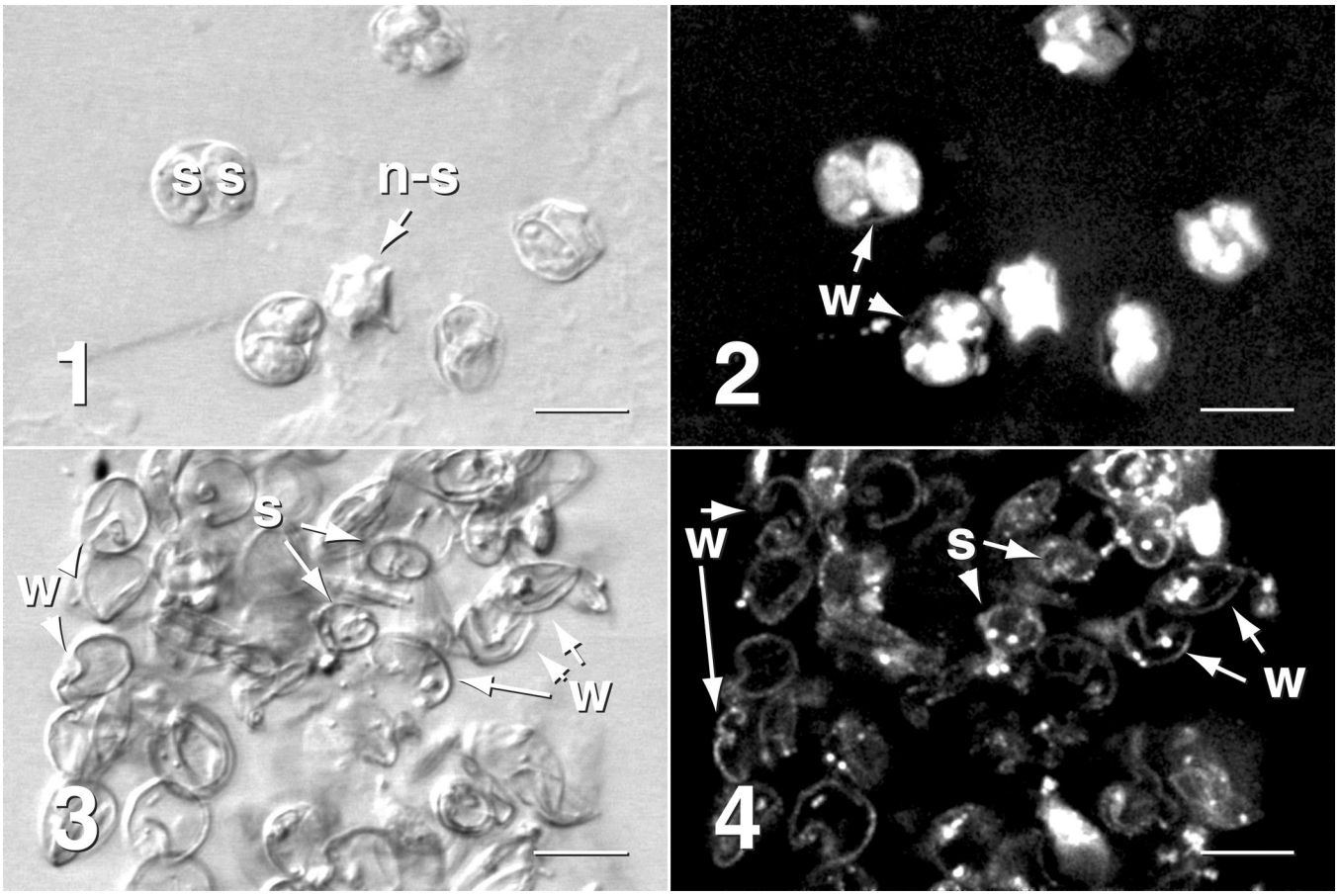

Fig. **1–4**. Comparison of differential interference contrast microscopy and Nile-Red fluorescence of intact oocysts vs. fragmented oocysts of *Toxoplasma gondii*. Bar = 10 μm. **1.** DIC microscopy of intact oocysts by laser scanning confocal microscopy, direct transmission, 488 nm line. **2.** The fluorescent image in the same plane as Fig. 1 taken by laser scanning confocal microscopy of the red fluorescence (Nile Red stained oocysts). The oocyst wall (w), sporocyst wall (s), and additional structures in sporozoites all exhibit fluorescence. The non-sporulated oocyst (n-s) exhibits a number of fluorescent structures. **3.** DIC microscopy of fragmented oocysts. **4.** The fluorescent image in the same plane as Fig. 3 taken by laser scanning confocal microscopy of the red fluorescence (Nile Red stained fragmented oocysts). These images were taken from a sample with less than optimal fragmentation, showing the spectrum of fragments obtained following vortexing of a sample with glass beads. The following objects are evident: a few intact oocysts, some walls still associated with single sporocysts, large wall fragments, intact sporocysts, a few broken sporocysts and a small amount of debris less than 1–2 μm in diam. Unbroken walls (both oocyst walls and sporocyst walls) revealed a continuous structure, whereas fragmentation was readily identified by breaks in the structure.

25 and 30% (v/v) iodixanol-sucrose in 15-ml polystyrene centrifuge tubes. Oocyst walls resolved at the interface between 25% and 30% (v/v) iodixanol, in both sedimentation and flotation gradients. In a representative experiment, the oocyst fraction contained 98% oocyst walls, 1% broken sporocyst walls, and 1% small fragments that could not be identified. The band was visibly less dense when fragments were top-loaded compared to when bottom-loaded, but both tubes contained 98% or greater purity of oocyst walls. Thus sedimentation gradients may produce a lower yield of oocysts than flotation gradients. The material that banded at the 25% to 30% (v/v) iodixanol interface contained oocyst walls (Fig. 5, 6).

The band of intact sporocysts was collected from the interface between 5% and 15% (v/v) iodixanol after 1 h of centrifugation, from both sedimentation gradients and flotation gradients (Fig. 7, 8). The yield of sporocysts from sedimentation gradients was higher than in flotation gradients, based on the visible density of the band at the interface, and confirmed by microscopic examination. However, in sedimentation gradients, the fraction was contaminated with a few non-sporulated oocysts, which were not present in this band in flotation gradients. In flotation gradients, non-sporulated oocysts were found scattered throughout fractions collected below the 5% and 15% (v/v) interface, indicating that non-sporulated oocysts moved more slowly than sporocysts and thus, sporocysts reached the interface between 5% and 15% after 1 h of centrifugation, whereas the non-sporulated oocysts had not reached this point after only one hour.

With respect to loading capacity, we loaded as many as 4×10^7 oocysts (prior to fragmentation) or as few as 1×10^6 oocysts and could separate clean fractions of sporocysts or walls under this wide range of concentrations (results not shown).

Separation by FACS. Based on the profiles from scans of forward scatter plotted against red fluorescence following Nile Red staining, we selected two regions that allowed us to separate oocyst walls from sporocysts by FACS (Fig. 9). Purity of fractions was determined by microscopic examination of the collected, sorted fractions, using DIC or fluorescence microscopy. Two fractions were obtained, one highly enriched in oocyst walls, and one highly enriched in sporocysts. Both fractions were of high purity (> 99/100 objects corresponded to

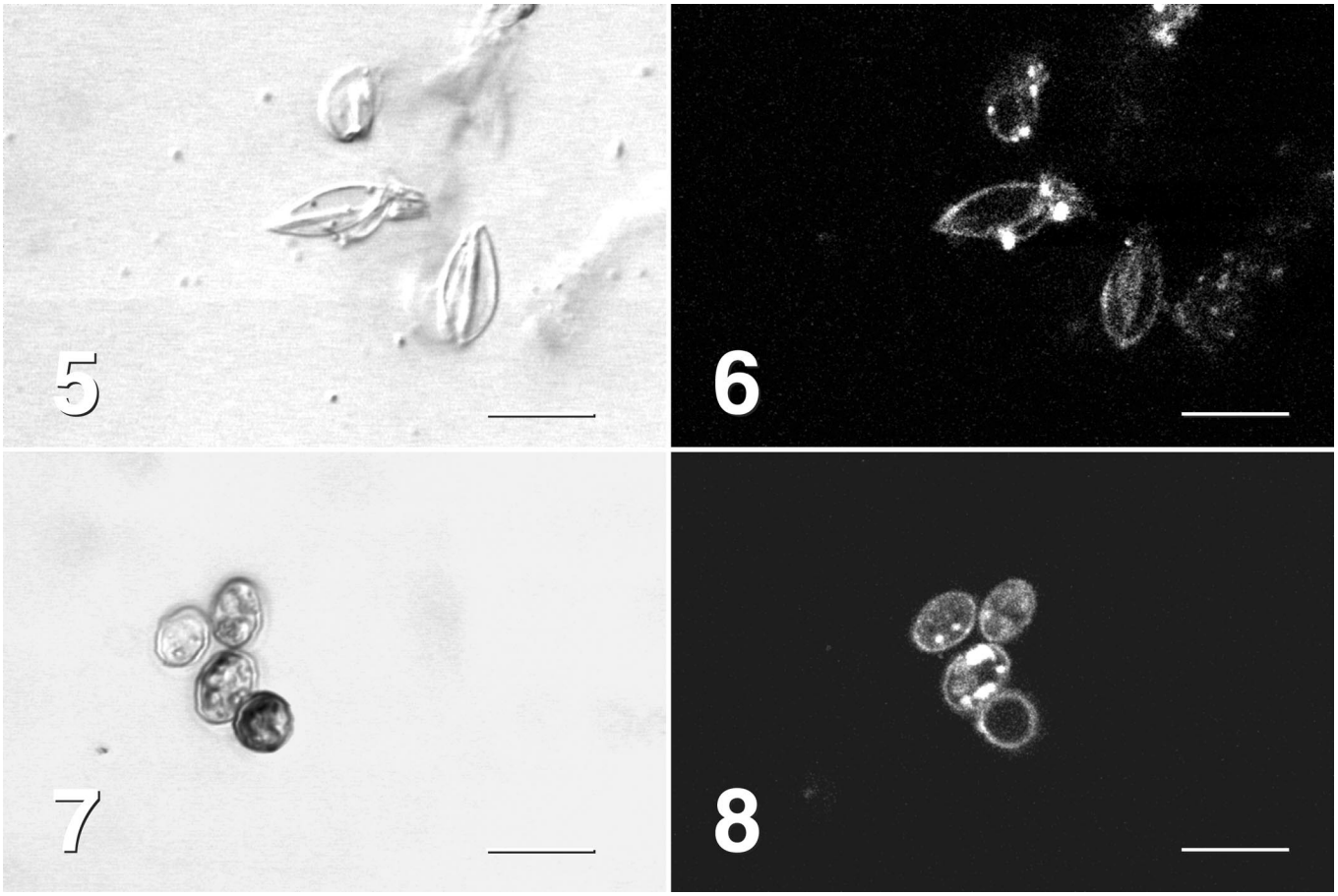

Fig. **5–8**. Purified fractions of fragmented oocysts of *Toxoplasma gondii* after iodixanol gradient centrifugation. Bar = 10 μm. **5.** Differential interference contrast microscopy of oocyst walls, obtained at the 25–30% (v/v) interface on iodixanol gradients. **6.** The corresponding image to Fig. 5 taken by laser scanning confocal microscopy of the red fluorescence following Nile Red staining. Note the wall fluorescence and associated punctate fluorescence. **7.** DIC microscopy of intact sporocysts, obtained at the 5–15% (v/v) interface on iodixanol gradients. **8.** The corresponding field to Fig. 7 taken by laser scanning confocal microscopy of the red fluorescence following Nile Red staining. Note both the wall fluorescence and the evidence of intracellular fluorescence corresponding to sporozoite membranes and lipid droplets.

either oocyst walls or sporocysts, respectively, by manual counting).

Confirmation of purity of fractions obtained by iodixanol gradient sedimentation using FACS. To confirm the purity of fractions obtained from iodixanol step gradients, pooled fractions of oocyst walls or of sporocysts were obtained following iodixanol gradient sedimentation, and then subjected to sorting by FACS. FACS allowed for independent confirmation of the purity by a non-microscopic method. In a representative experiment, the fraction from the 25% to 30% iodixanol interface (i.e. the oocyst wall fraction) had a purity of 100% oocyst wall fragments: all particles were sorted within the large oocyst wall region entirely. The fraction from the 5% to15% interface (i.e. the sporocyst fraction) showed 98% sporocysts and 2% non-sporulated oocysts.

DISCUSSION

The effectiveness of the fragmentation procedure is a critical step in the isolation of oocyst walls. We established criteria for fragmentation that yielded good separations of oocyst walls and sporocysts. We found that visible disruption of the oocyst wall was not sufficient, but that mechanical processing had to continue until the the majority of sporocysts were released from oocyst walls. If this treatment did not occur, the oocyst wall fractions became contaminated with partially fragmented oocysts (oocyst walls with sporocysts still attached). Thus, the lighter buoyancy of a single sporocyst attached to a denser oocyst wall is insufficient to change the sedimentation or flotation properties to allow these two components to separate. Upon separation, however, unattached sporocysts exhibited a large difference in buoyancy from oocyst walls.

The proportion of beads to oocysts appeared to be an important criterion for successful fragmentation. Increasing the number of glass beads per tube led to excessive fragmentation of wall fragments, breakage of sporocysts, and excessive small debris without release of sporocysts from oocyst walls. Increasing the concentration of oocysts resulted in incomplete fragmentation even if the time of fragmentation was prolonged. Excessive fragmentation resulted in poor recovery and contamination of all fractions by small debris. In addition, excessive fragmentation caused contamination of fractions containing oocyst walls with additional fragments (i.e. broken sporocysts and sporocyst walls and small unidentified fragments of debris).

The studies reported in this paper describe the first set of methods for isolation of oocyst walls and sporocysts. Percoll gradient separation allowed for isolation of a fraction enriched in sporocysts within the shortest time frame. The total time for fragmentation and Percoll gradient separation of sporocysts was

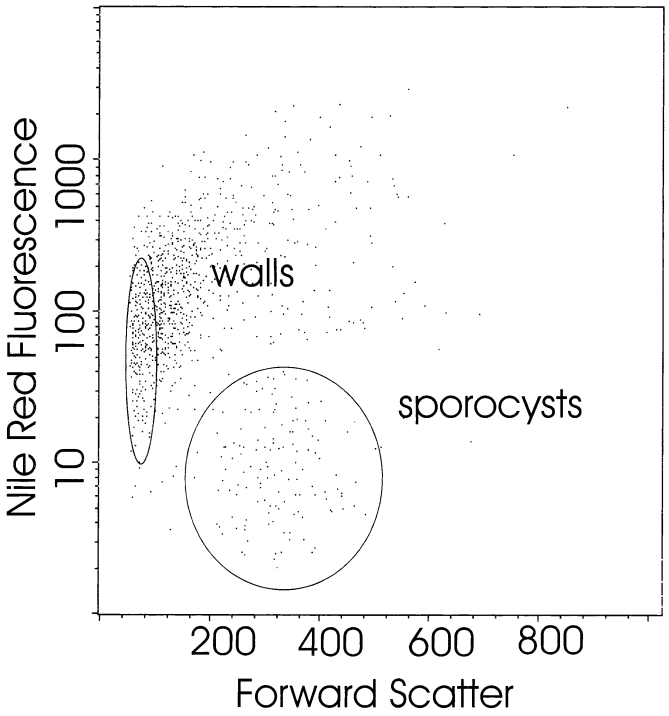

Fig. **9**. Flow-cytometry profile of fragmented oocysts of *Toxoplasma gondii*. Fragmented oocysts were sorted by flow cytometry, using Nile Red fluorescence (vertical axis, arbitrary units on a logarithmic scale) vs. forward scatter (horizontal axis, arbitrary units on a linear scale) as the basis for development of the profile. Nile Red permeated and became fluorescent within both oocyst walls and sporocysts. The two regions which were used to identify and sort oocyst walls (left, oval) and sporocysts (right, circle) are plotted on the profile.

45 min. Sedimentation on iodixanol resulted in the isolation of material from as many as 4×10^7 oocysts per gradient tube (starting concentration), a quantity suitable for studies requiring larger amounts of purified material. FACS was useful for obtaining high purity fractions even when fragmentation conditions were not optimal.

The availability of oocyst walls and sporocysts will facilitate a number of further investigations. Oocyst walls are an enriched starting material for development of detection methods, for isolation and characterization of the wall composition. Sporocysts can be useful for examination of excystation, for use in proteomic studies to identify proteins unique to this life cycle stage, and in studies of the process of invasion. The use of inert materials (iodixanol gradients) in the preparation of sporocysts eliminates steps in removal of materials toxic to the sporozoite or to cells in culture, prior to further studies on excystation or invasion, simplifying the methods for such studies.

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